Urokinase Stimulates Differentiation of Fibroblasts into Myofibroblasts and Their Proliferation in Damaged Adventitia

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Urokinase expression in the adventitia of rat common carotid artery increased on the 4th day after periadventitial damage. Periadventitial application of recombinant urokinase increased the area of the adventitia and the content of contractile and proliferating cells, while proteolytically inactive recombinant urokinase was ineffective.

Key Words: *urokinase; adventitia; fibroblasts; proliferation*

Remodeling of the vascular wall is a critical stage in the pathogenesis of various vascular diseases, including atherosclerosis and restenosis [15]. Adventitial fibroblasts are involved in vascular wall remodeling after balloon angioplasty [14] and during progressive arterial hypertension [1]. Activated fibroblasts are differentiated into myofibroblasts, proliferate, and migrate to other layers of the vascular walls [9]. Growth factors, cytokines [15], and proteases, in particular plasmin, play the major role in regulation of these processes. Plasmin is serine protease with wide specificity, which is formed from plasminogen under the effect of two specific tissue- and urokinase-type activators. It was shown that processes involving cell migration (reparation, inflammation, and angiogenesis), are accompanied by the activation of extracellular proteolysis predominantly mediated by urokinase-type plasminogen activators [11,12]. Urokinase contributes not only to cell migration, but also stimulates cell proliferation due to proteolytic activation of latent and matrix-bound growth factors (indirect stimulation) or due to the presence of growth domain in its structure (direct stimulation) [2,6]. Chemotactic and mitogenic effects of urokinase were studied on cultured cells [10]. *In vivo* experiments showed that urokinase and its receptor are expressed in atherosclerotic plaques and neointima formed after balloon angioplasty [5,11]. During wound healing, myofibroblasts express urokinase [3] and its receptor [8]. However, the effects of urokinase on transformation of fibroblasts into myofibroblasts and their proliferation and migration to the adventitia after damage are poorly understood. Here we studied the effects of urokinase on transformation of adventitial fibroblasts into contractile cells, their proliferation, and remodeling of the adventitia after periadventitial damage *in vivo*.

MATERIALS AND METHODS

Experiments were performed on male Wistar rats. Periadventitial damage to the left common carotid artery (CA) was induced under ketamine anesthesia (10 mg/kg). Native recombinant urokinase (20 nmol/kg) [10] dissolved in 0.5 ml 40% Pluronic F-127 gel [13] was applied to damaged adventitia in 7 animals. To estimate the proteolytic activity of urokinase, 5 animals were treated with proteolytically inactive modified prourokinase, in which histidine-204 in the active site was substituted by glutamine [4]. Seven control rats were treated with 0.5 ml Pluronic F-127 gel. Four days

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postoperation, the rats were anesthetized with 2 mg/kg pentobarbital and perfused with 4% formaldehyde through the left ventricle at 120 mm Hg for 10 min. Before fixation, the vessels were washed with 0.9% NaCl at 120 mm Hg for 2 min. CA was isolated, dehydrated in increasing ethanol concentrations, chloroform-ethanol mixture, and chloroform, and then embedded into paraplast (Sigma). Slices (4.5 and 10 μ) were prepared for immunohistochemical and morphometrical assays, respectively.

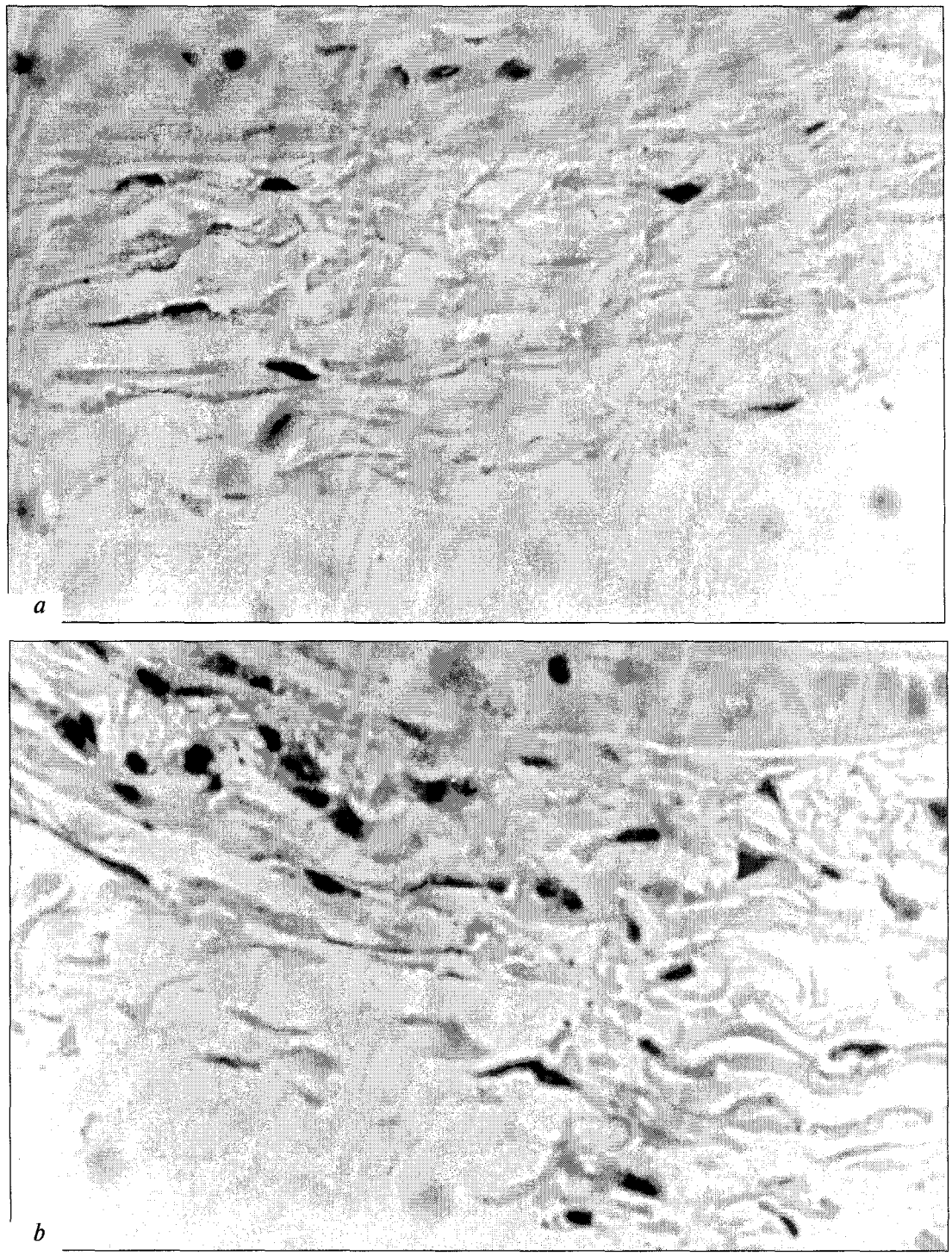

Fig. 1. Expression of urokinase in the adventitia of rat common carotid artery after periadventitial damage: intact vessel (*a*) and day 4 after damage (*b*). Immunohistochemical staining. Cell nuclei are stained with hematoxylin and eosin, ×480.

TABLE 1. Changes in the Adventitia of the Common CA 4 Days after Application of Recombinant Urokinases ($M\pm m$, n=5)

Parameter	Intact vessel	Control	Urokinase	
			active	inactive
α-Actin-positive cells, %	6.76±1.47	24.98±6.80*	55.71±10.40+	30.0±8.7°
Proliferation index, %	5.1±2.5	11.9±1.0	51.2±6.1+	14.4±1.4°
Change in the area of the adventitia, mm²	—	-0.068±0.048	0.999±0.200+	0.111±0.140°

Note. $p<0.05$: *compared to intact vessel; +compared to the control; °compared to active urokinase.

For morphometry, slices were deparaffined with xylene and decreasing ethanol concentrations, stained with 0.1% toluidine blue, dehydrated by immersion (standard procedure), and then embedded into Canada balsam (Sigma). Morphometry of CA slices was performed using KS-100 2.0 software (Kontron Elektronik GmbH), and the areas of the media and adventitia and the lumen of CA were measured using Optimas 6.1 software (Optimas Corporation).

Immunohistochemical staining was conducted using polyclonal rabbit antibodies to human urokinase (Inpharm), polyclonal mouse antibodies to smooth muscle α-actin (Sigma), monoclonal mouse IgG to proliferating cell nuclear antigen (PCNA, Santa Cruz Biotechnology), goat serum (ICN), and second biotinylated horse anti-mouse immunoglobulins (Inpharm). Antibodies bound to target proteins were visualized by the reaction of avidin—biotin—horseradish peroxidase (Vector laboratories) with diaminobenzidine hydrochloride (Sigma). Immunohistochemical visualization of urokinase, α-actin, and PCNA was performed by routine methods. The slices were deparaffined with xylene and decreasing ethanol concentrations; endogenous peroxidase activity was inhibited with 3% H_2O_2 (20 min). The slices were incubated with 10% blocking serum for 30 min and with primary antibodies to urokinase (dilution 1:200) and α-actin and PCNA (dilution 1:50) in 10% blocking serum for 60 min. Control slices were incubated with 10% blocking serum. Incubation with secondary antibodies in 10% blocking serum (dilution 1:200) for 60 min was followed by 30-min incubation with the complex of avidin—biotin peroxidase (dilution 1:100) in 10% blocking serum and phosphate buffer. Diaminobenzidine tetrahydrochloride (0.5 mg/ml in 0.01% H_2O_2) was added for 2-4 min. The slices were stained with hematoxylin and eosin, dehydrated with ethanol and xylene, and embedded in Canada balsam (Sigma).

PCNA-positive ($PCNA^+$) and PCNA-negative ($PCNA^-$) cells were counted. The index of proliferation was calculated by the formula:

$$I=PCNA^+/PCNA^+ + PCNA^-.$$

The ratio of α-actin-positive cells was calculated similarly. The results were analyzed by Student's t test at p=0.05 and expressed as arithmetic mean±standard error.

RESULTS

Expression of endogenous urokinase considerably increased after periadventitial damage both in the adventitia and media (Fig. 1, *b*), but was absent in intact vessels (the right common CA, Fig. 1, *a*). The index of proliferation in damaged adventitia also increased and was 28.5±2.8 *vs.* 11.8±1.2% in intact vessels ($p<0.05$). Fibroblast activation and differentiation into myofibroblasts are necessary for their proliferative response [7]. Damage to the adventitia and application of Pluronic F-127 gel increased the content of phenotypically contractile cells (α-actin-positive cells) on day 4 postoperation ($p<0.05$ compared to intact vessels, Table 1) and the area of the adventitia ($p<0.05$ compared to intact vessels, data not shown).

Hence, periadventitial damage activates expression of urokinase in the adventitia, stimulates proliferation of adventitial cells, increases the content of α-actin-positive cells (which indicates transformation of fibroblasts into myofibroblasts) and the area of the adventitia. Expression of endogenous urokinase is not necessarily related to proliferation and transformation of adventitial cells after damage. However, our findings suggest that urokinase is involved in the regulation of vascular remodeling [9].

To evaluate the role of urokinase and its proteolytic function we studied the effects of periadventitial application of proteolytically active and inactive recombinant urokinases on the index of proliferation, content of α-actin-positive cells in the adventitia, and area of the adventitia. Native urokinase significantly elevated these parameters ($p<0.05$, Table 1), while proteolytically inactive urokinases produced no changes compared to control rats treated with Pluronic F-127 gel (Table 1).

The total number of adventitial cells increased after application of proteolytically active urokinase (243.5±26.5 *vs.* 165.5±8.1 in the control, $p<0.05$),

while proteolytically inactive urokinase had no effect on this parameter (170.5±25.5).

Our findings indicate that urokinase is involved (primarily due to its proteolytic activity) in fibroblast activation and differentiation to myofibroblasts and potentiated proliferative response to damage in the adventitia. These data suggest that modulation of urokinase expression in damaged vascular wall holds much promise for the regulation of vascular wall remodeling during restenosis and atherosclerosis.

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